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Detection and identification of a 16SrIII-J phytoplasma affecting cassava (*Manihot esculenta* Crantz) in Argentina

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Abstract

Cassava plants displaying symptoms of dwarfing, witches' broom, and chlorosis, were detected in production fields located in Misiones province Argentina. The RFLP profiles and phylogenetic analyses based on 16S rRNA and ribosomal protein (*rplV-rpsC*) gene sequences, allowed assignment of the phytoplasma detected in cassava to the 16SrIII-J subgroup. This is the first report of a phytoplasma affecting cassava in Argentina.

Keywords Phytoplasma · Cassava · Yellowing · Sequence · Argentina

Cassava is grown across tropical regions in South America, Africa and Asia and it is a major staple food for an estimated 800 million people (FAO 2013). In 2013, Argentina produced about 180,000 tons of cassava focusing its production in the Northeast region (NE). Within this region, the Misiones province has the largest cultivated area (18,000 ha) and is responsible for about 70% of the country's total production (Aristizábal and Calle 2015). Several phytoplasmas have been reported infecting cassava in other regions of the world; these include those associated with cassava frog skin disease (CFSD) (Alvarez et al. 2009) and cassava witches' broom (CWB). For CWB, phytoplasmas from groups 16SrI (Arocha et al. 2009; Alvarez et al. 2013), 16SrII (Trinh et al. 2015) and 16SrIII (Flôres et al. 2013) have been described in association with symptoms such as dwarfism, leaf proliferation, shortened internodes and, at times, smaller roots. During 2017, cassava plants with symptoms of dwarfism, witches' broom and chlorosis were observed in cassava production lots located in Misiones province (Fig. 1). The authors are not aware of any previous reports of phytoplasmas affecting cassava crops in Argentina, so the aim of this work was to detect and identify the pathogen associated with the described symptomatology and undertake its molecular characterization. To achieve this, cassava symptomatic (4) and symptomless (2) plants were collected from one plot located in Puerto Rico (Misiones Province). For DNA isolation, petioles and midribs were ground in liquid nitrogen according to the methods of the Doyle and Doyle (1990) DNA extraction protocol. Phytoplasma detection was performed by PCR amplification of the 16S rRNA and *rplV-rpsC* genes using the primer pairs R16F2-R16R2 (1.2 kb) (Lee et al. 1993) and rpF1-rpR1 (1.3 kb) respectively (Lim and Sears 1992). PCR reactions were performed in solutions containing 100 ng of DNA, 0.4 mM of each primer, 200 mM of each dNTP, 1 U of GoTaq® DNA polymerase, 1X polymerase buffer (Promega, USA) and sterile water to a final volume of 40 μl. Thermal cycler conditions for primer pair R16F2-R16R2 (1.2 kb) and rpF1-rpR1 (1.3 kb) were 94 °C three minutes for initial denaturalization, thirty five cycles of 94 °C for 1 min, 54 °C for 2 min and 72 °C for 2 min, and final extension at 72 °C for 5 min. Amplification products of 1.2 kb and 1.3 kb were obtained in all symptomatic samples, while no amplifications were observed from the symptomless samples. The 1.2 kb amplified fragments were subjected to restriction digestion using *Mse*I, *Hha*I and *Rsa*I endonucleases (NEB, USA) according to the manufacturer's instructions. The actual RFLP profiles in all four positive samples were undistinguishable from those of the *Bellis virescence* phytoplasma (Galdeano et al. 2013) used as a reference pattern for the subgroup 16SrIII-J. For in silico RFLP and phylogenetic analyzes, two isolates were selected to be sequenced, CasDWB-Arg3

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Fig. 1 Symptoms of dwarfism, chlorosis (**a**), and witches' broom (**b**) in cassava plants observed in production fields in Misiones province. Yellow arrow: symptomatic plant. White arrow: asymptomatic plant

and CassDWB-Arg4 (CasDWB: Cassava Dwarfism Witches' Broom). Partial 16Sr RNA and complete *rplV-rpsC* genes sequences were obtained using an automated DNA sequencer (Unidad Genómica, Instituto de Biotecnología-INTA; Argentina). Consensus sequences were assembled using Geneious R10 (Kearse et al. 2012) and deposited in a public databank (NCBI) (Accession numbers MF563363 and MF563364 for 16S rRNA and MF563965 and MF563966 for *rplV-rpsC*).The 16Sr DNA group/subgroup affiliations were assigned by in silico RFLP profile analysis of the 16S rDNA sequence using the *i*Phyclassifier program (Zhao et al. 2009). The virtual collective RFLP patterns derived from the query 16S rDNA fragments were identical to the reference pattern of 16Sr group III, subgroup J (reference sequence: AF147706). These findings were corroborated by phylogenetic analysis using the Maximum Likelihood method with MEGA6 software (Tamura et al. 2013). The phylogenetic tree constructed based in 16Sr DNA gene sequences grouped the CassDWB isolates within the subgroup 16SrIII-J clade and clearly distinguished from the other 16SrIII subgroups (Fig. 2). Using the *rpLV-rpsC* gene sequences the final tree shows a topology similar to those described by others authors (Galdeano et al. 2013). The sequences of the *rpLV-rpsC* genes do not clearly resolve the subgroups III-J and III-B; however, they can clearly distinguish them from the rest of the 16SrIII-subgroups. The CassDWB isolates characterized in this work were grouped in the same clade with phytoplasmas from subgroups 16SrIII-B and 16SrIII-J (Fig. 3).

Phytoplasmas from subgroup 16SrIII-J have been reported previously in Brazil, Argentina and Chile (Montano et al. 2011; Fugita et al. 2017; Fernández et al. 2017; Quiroga et al. 2017) and recently a lineage related to this subgroup has been described in Mexico (Pérez-López et al. 2017).Plant species affected by these subgroup are diverse and include garlic (*Allium sativum*), chayote (*Sechium edule*), sunflower (*Helianthus annuus*), cauliflower (*Brassica oleracea*), eggplant (*Solanum melongena*), strawberry (*Fragaria* × *ananassa*), daisy (*Bellis perennis*), *Celosia* spp., periwinkle (*Catharanthus roseus*), *Aegiphila verticillata*, *Vernonia brasiliana*, lettuce and Swiss chard (*Beta vulgaris*). The CassDWB phytoplasma described in this works belongs

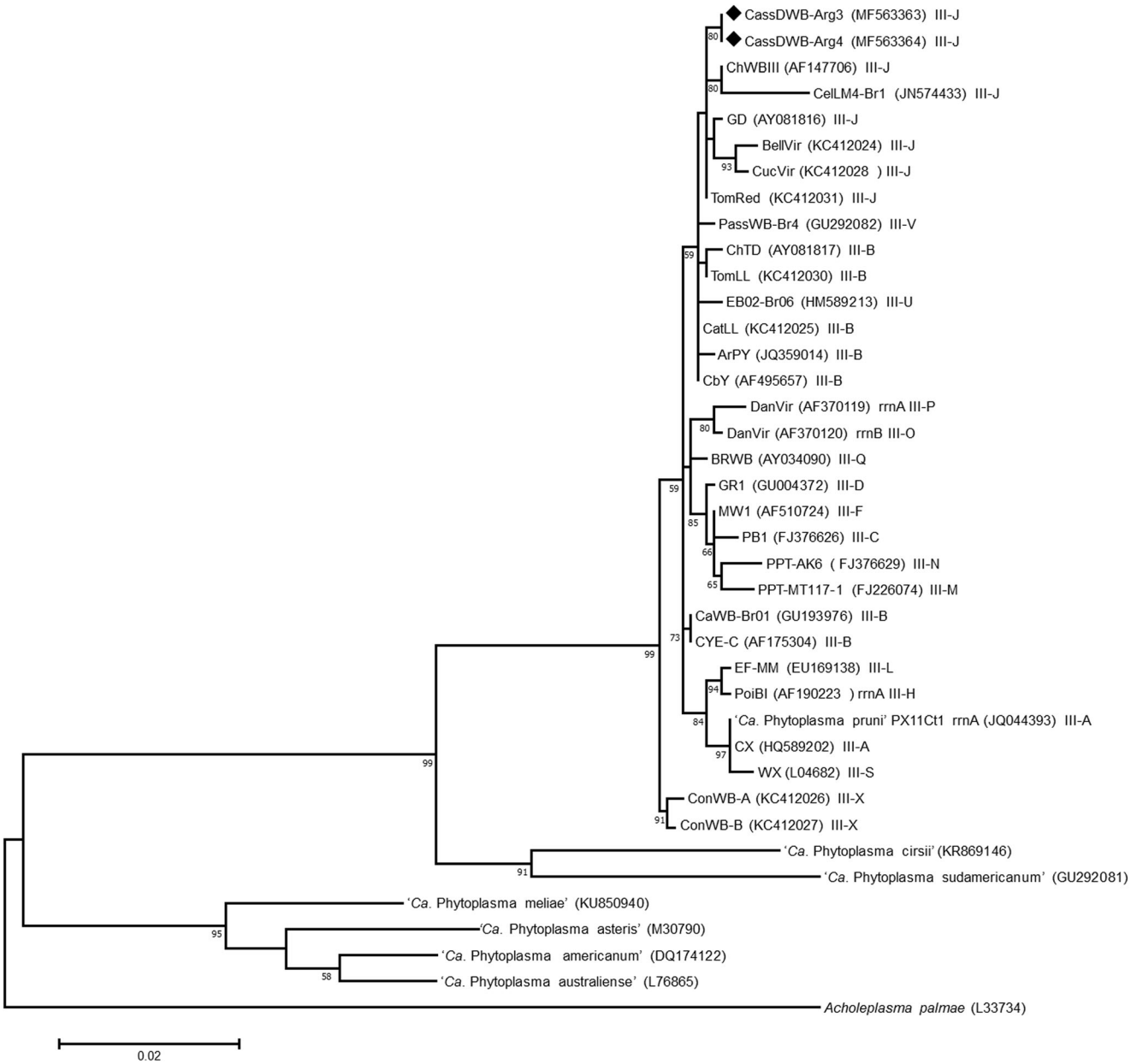

Fig. 2 Phylogenetic tree inferred from analysis of 16S rDNA sequences using the Maximum-Likelihood method. The GenBank accession number for each taxon is given in parentheses. The numbers on the branches are bootstrap (confidence) values (1000 replicates). CassDW sequences are marked with black diamonds. *Acholeplasma palmae* was used as out-group. The scale bar represents the number of nucleotide substitutions per site. The corresponding 16SrIII-subgroup was added to each taxon

to the 16SrIII-J subgroup and to the authors' knowledge constitutes the first report of a phytoplasma affecting cassava in Argentina. This is also believed to be the first report of a 16SrIII-J subgroup affecting cassava worldwide. The detection and identification of the CassDWB phytoplasma in the NE region of Argentina extends the distribution of this subgroup and increases the knowledge about its host range. In previous work, Flôres et al. (2013) describes a phytoplasma from the 16SrIII-B subgroup associated with cassava witches' broom disease in Brazil. Cassava frogskin disease (CFSD) was also reported in Colombia (Alvarez et al. 2009) to be associated with a phytoplasma from subgroup 16SrIII-B. Characteristic CFSD symptoms in the roots are a woody aspect and a thickened peel that is cork-like, fragile, and opaque (Alvarez et al. 2009). No symptoms of CFSD were observed in samples infected with CassDWB phytoplasma. It is necessary to continue studying this

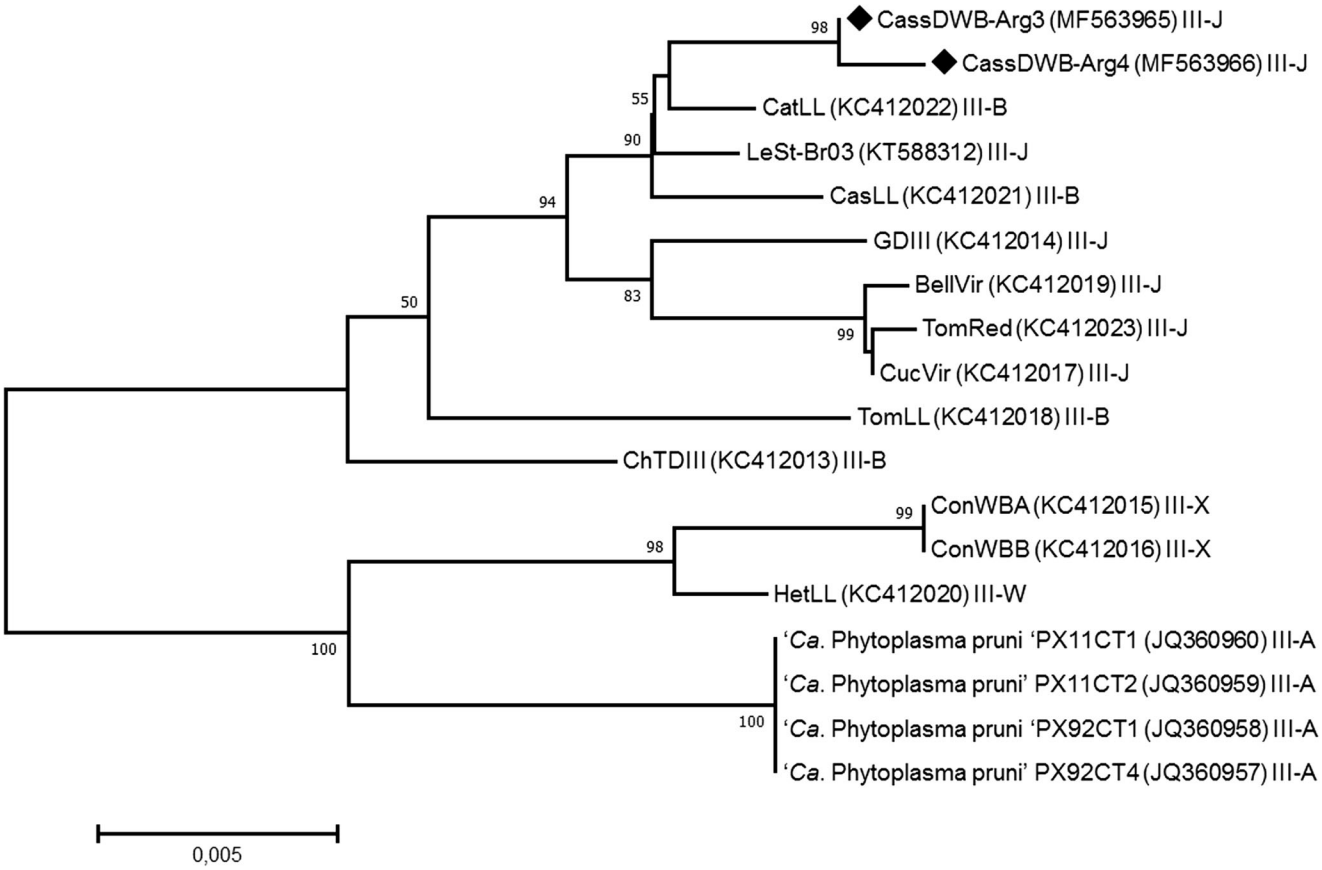

Fig. 3 Phylogenetic tree inferred from analysis of *rpLV-rpsC* nucleotide sequences using Maximum-Likelihood method. The GenBank accession number for each taxon is given in parentheses. The numbers on the branches are bootstrap (confidence) values (1000 replicates). CassDW sequences are marked with black diamonds. The scale bar represents the number of nucleotide substitutions per site. The corresponding 16SrIII-subgroup was added to each taxon

pathosystem, identifying vectors and natural reservoirs as well as evaluating the behavior of different cassava germplasm to achieve a more efficient management of the disease. It is also essential to improve the detection of infected material in Argentina as well as the material that is introduced into the territory, particularly as this is a clonally multiplied crop.

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